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Primate-specific endogenous retrovirus driven transcription defines naïve-like stem cells

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Naïve embryonic stem cells (ESCs) hold great promise for research and therapeutics as they have broad and robust developmental potential. While such cells are readily derived from mouse blastocysts it has been impossible to easily isolate human equivalents^{1,2}, although human naïve-like cells have been artificially generated (rather than extracted) by coercion of human primed ES cells by modifying culture conditions²⁻⁴ or through transgenic modification⁵. Here we show that a sub-population within cultures of human ESCs (hESCs) and induced pluripotent stem cells (hiPSCs) manifest key properties of naïve state cells. These naïve-like cells can be genetically tagged, and are associated with elevated transcription of HERVH, a primate-specific endogenous retrovirus (ERV). HERVH elements provide functional binding sites for a combination of naïve pluripotency transcription factors, including LBP9, recently recognized as relevant to naivety in mice⁶. LBP9/HERVH drives hESC-specific alternative and chimeric transcripts, including pluripotency modulating long non-coding RNAs (lncRNAs). Disruption of LBP9, HERVH and HERVH-derived transcripts compromises self-renewal. These observations define HERVH expression as a hallmark of naïve-like hESCs, and establish novel primate-specific transcriptional circuitry regulating pluripotency.

While many genes are involved in pluripotency, transposable element (TE) transcription, particularly involving ERVs, has wired different genes into the network in humans and mice⁷. Given a role for ERVs in pluripotency⁸⁻¹⁰, we surveyed RNAseq data of human pluripotent stem cells (hPSCs), notably hESCs and hiPSCs finding that several TEs are expressed at higher levels in hPSCs, ERV1

type of long terminal repeat (LTR) retroelements being foremost, of which HERVH was the most highly expressed^{8,11} (Figs. 1a-b, E1a-b). Uniquely aligned reads (Table S6) indicate that 550 of the 1225 full-length HERVH genomic copies are transcribed in hPSCs (Figs. E1c-d; Table S7). Raised transcription was associated with elements containing consensus LTR7 rather than diverged variants (LTR7B/C/Y: Table S7). Lower expression of other ERVs (Fig. 1b) was confirmed via qRT-PCR (Fig. 1c). We focused on HERVH as this was the only one detected by qRT-PCR in all hiPSC lines analysed (Fig. 1c). Results are robust to use of reads that map to more than one location (Table S16).

To address how specific HERVH transcription is to hPSCs we compared RNAseq datasets of hPSCs and multiple differentiated cells and tissues (Fig. E1c; Tables S4, S5, S7). In agreement with our hiPSC data, HERVH transcription was highest in hPSC lines. The majority of the transcribed loci are identical between hiPSCs and hESCs (Figs. E1c-d). HERVH transcription levels are much lower in both differentiated cells and cancer cell lines (Fig. E1c).

HERVH transcription levels are higher in hiPSCs at early passages following reprogramming (Fig. 1d), indicating that the reprogramming process itself might induce HERVH expression. At later passages the transcription of HERVH in hiPSCs approaches hESC levels.

Consistent with HERVH transcription in hPSCs, ChIP-seq data show that, in contrast to HERVK and inactive HERVHs, active HERVHs are marked with transcriptionally active histone marks^{11,12} (H3K4me1/2/3, H3K9ac, H3K36me3

and H3K79me2), while the repressive marks (H3K9me3 and H3K27me3) are rare, indicating functioning as active promoter/enhancers (Figs. 2a, E2a-e). Notably, active HERVHs are also enriched with binding sites of the pluripotency regulators/modifiers CHD1¹³ and Myc/Max¹⁴ (Figs. E2b-c; Table S15). HERVH activation is also inversely correlated with the DNA methylation status of LTR7 of HERVH, as evidenced by hypomethylation in active LTR7 regions in hPSCs¹⁵ (Fig. E2f).

To determine whether HERVH is a direct target of core pluripotency-associated transcription factors (TFs) we interrogated HERVH in hESC_H1 ChIP-Seq data³. This identified NANOG and OCT4 (Fig. E3a). A candidate KLF4 binding site was also identified within HERVH's LTR (Fig. 2b). We additionally asked which TF motifs are significantly enriched across four *in silico* tests (Fig E3b). Only one, LTR-binding protein 9 (LBP9) – alias murine Tfcp2l1 - was significant across all analyses (Fig. E3b). Tfcp2l1 is within the Oct4 interactome¹⁶ and binds regulatory regions of Oct4 and Nanog¹⁷ in mESCs. LBP9's direct binding to LTR7 is confirmed by ChIP-qPCR and EMSA (Fig. 2c, and Fig. E3c). EMSA further demonstrates LBP9/NANOG cooperation in binding LTR7 (Fig. E3c), consistent with synergy following simultaneous over-expression (Fig. E7c). LBP9-specific binding was also detected in the 5'-region of NANOG (Fig. 2c).

In vitro differentiation assays show that HERVH transcription levels decline over time in parallel with declines in OCT4, NANOG and LBP9 (Fig. E3d), suggesting a role in HERVH expression. As expected, ectopic expression of LBP9, OCT4, NANOG and KLF4 activated the pT2-LTR7-GFP#2 reporter and enhanced

endogenous HERVH transcription levels in human primary fibroblast (HFF-1), while overexpression of c-MYC or SOX2 had no effect (Fig. 2d, E7c). Conversely, a complementary 'loss of function' RNAi assay in hESCs_H9 revealed that HERVH transcription levels were reduced following OCT4, NANOG and LBP9, but not SOX2, knockdown (KD) (Figs. 2e-f).

We confirmed that LBP9 directly stimulates HERVH-driven expression, by comparing in hiPSCs signals of a wild-type (WT) pT2-LTR7-GFP#1 reporter construct and a mutant lacking the LBP9 motif (Δ LBP9: Fig. E7d). When WT and mutant constructs were transfected into hiPSCs, the GFP signal was clearly detected from the WT reporter, but it was decreased by 2-fold in Δ LBP9 (Fig. E7d).

ESC-specific TFs OCT4, NANOG, KLF4 and LBP9 thus drive transcription in hPSCs. In contrast to mice in which LBP9 binding sites are genomically distinct from those other pluripotency TFs⁶, the key pluripotent TFs cluster within the primate-specific HERVH (Fig. 2b).

To test the functional importance of HERVH, we analysed RNAseq data to investigate the influence of LTR7/HERVH on the expression of neighbouring regions. We find that LTR7 initiates chimeric transcripts, functions as an alternative promoter or modulates RNA processing from a distance (Figs. 3a, E4b; Tables S8-9). 128 and 145 chimeric transcripts were identified in hiPSCs and hESCs, respectively (Fig. E4a; Tables S8,-9). One gene can contribute to multiple chimeric transcripts. The chimeric transcripts between HERVH and a

downstream gene generally lack the 5' exon(s) of the canonical version (e.g. SCGB3A2) while part of HERVH/LTR7 is exonized (e.g. RPL39L) (Fig 3a). A significant fraction of HERVH sequence can be incorporated into novel, lineage-specific genes (e.g. ESRG: Fig. 3a) or lncRNAs (e.g. RP11-69I8.2: Fig. E4d and Table S10). We confirmed several hPSC specific chimeric transcripts by RT-PCR (Fig. 3a). Transcriptional start signals commonly map to HERVH-LTR boundary regions (Fig. E4c). Unlike the chimeric transcripts the canonical genes are commonly not expressed in pluripotent cells.

Nearly 10% of the transcripts driven off HERVH are annotated as lncRNA¹² (see Table S11 for coding potential). 54 transcripts were identified that are commonly detected in hPSCs, while the rest were sporadic (Fig. E4d). The former set includes linc-ROR and linc00458, known to modulate pluripotency^{18,19}. Alignment of the 22 most highly expressed transcripts reveals an LTR7/HERVH-derived conserved core domain (CD) (Fig. E4f). The domain is predicted to bind RNA-binding proteins, including pluripotency factors (e.g. NANOG) and pluripotency-associated histone modifiers (e.g. SET1A and SETDB1) (Fig. E4g). In agreement with a role in pluripotency, linc00458 physically interacts with SOX2¹⁹.

To explore the effect of either LBP9 or specific HERVH-derived transcripts on the reprogramming process, we asked whether forced expression of LBP9, ESRG or the conserved domain of lncRNAs (LTR7-CD) modulates the fibroblast-hiPSC transition. While the overexpressed gene products affect neither pluripotency

nor self-renewal (Figs. E5a-b), all facilitate reprogramming by accelerating the mesenchymal-epithelium transition or hiPSC maturation (Figs 3b, E5c).

While LBP9 is key to the murine naïve state^{6,20}, HERVH is primate-specific. To determine whether HERVH/LBP9 delineates a primate-specific pluripotency circuitry, we performed “loss of function” experiments using small hairpin RNAs (shRNAs) against LBP9 or HERVH (Figs. 3c-f, E5d-g). Pluripotency-associated TFs and markers are down-regulated, while multi-lineage differentiation markers are up-regulated upon knockdown of either, but not in controls (Fig. 3c-d, E5f-g). Depletion of LBP9 or HERVH in hESCs thus results in loss of self-renewal. Knockout of LBP9 similarly abolishes hESC self-renewal (Figs. E5h-j). In contrast to hPSCs, the Tfcp2l1/LBP9 knockdown in mESCs does not reduce levels of Oct4, Sox2 and Nanog in serum-based conditions (Fig. E5k)²¹, but only in 2i⁶. In fact, Tfcp2l1/LBP9 does not affect self-renewal, but rather differentiation potential (Fig. E5k).

Genome-wide gene expression patterns are highly similar between LBP9 and HERVH knockdowns (Fig. 3e), consistent with LBP9 regulating HERVH-driven expression. 1094 of the 2627 genes are similarly regulated in LBP9/HERVH knockdowns (Fig. 3f; Table S12). While some HERVH-derived chimeric transcripts are potentially directly affected by depletion of HERVH (Tables S13-14), qRT-PCR identifies 19 HERVH-derived lncRNAs, down-regulated in response to both HERVH and LBP9 knockdowns (Fig. E4e).

While several of the differentially expressed genes are associated with murine pluripotency, the LBP9/HERVH-driven list of transcripts defines a primate-specific pluripotency network. Our analyses defined two classes of genes, (I) those conserved between mouse and human that contribute to the pluripotency in both, and (II) a primate-specific group that includes (a) those with an orthologous partner, but are not involved in murine pluripotency and (b) novel (not in mouse) transcripts (Figs. E4b, E4d). Several HERVH elements in class IIa affect gene expression in *cis*, and drive specific genic isoforms (e.g. SCGB3A2). A subset of class IIb contains HERVH-derived novel sequences (e.g. linc-ROR, linc000548, ESRG) (Fig. E4d).

We examined one class IIb transcript in detail. ESRG has a putative open reading frame (ORF) only in human (Fig. E6a; Supplementary Data 1), and is uniquely expressed in human inner cell mass (ICM) and PSCs (Fig. E6b). Knockdown of ESRG compromised self-renewal of hESCs, as many pluripotency-associated genes were decreased, while SOX2 expression was slightly elevated (Figs. E6c-e). The KD-ESRG colonies lost their hESC morphologies and committed to differentiation (Figs. E6e-f). Expression of ESRG along with the OSKM pluripotency factors has a similar effect on the reprogramming process compared with LBP9 (Fig E5c). ESRG is thus an HERVH-associated novel gene required for human-specific pluripotency, with a more specific phenotype than upstream regulators.

Given that the naïve-associated TFs together cluster on HERVH and the HERVH-derived products are essential for primate pluripotency, we asked whether

HERVH-driven transcription marks the naïve-like stage in hPSC cultures. To explore this the reporter construct, pT2-LTR7-GFP#2 was integrated into the genome of either mouse or human PSCs (Figs. 4a, E7a-b, E8i) by *Sleeping Beauty* gene transfer, providing stable transgene expression²². While all of mESC colonies homogeneously express GFP (Fig E7a), only ~4% of cells in each hESC colony show a strong GFP signal (GFP(high)), indicating cellular heterogeneity (Figs. E7e, E7h-j). The fraction either weakly or unexpressing GFP we term GFP(low) and GFP(-) respectively (Fig 4a, E7b, E7e). RNAseq data of hESCs from single cells^{23,24} and hPSC lines confirm that pluripotent cultures exhibit variability in HERVH expression (Fig. E1d), indicating that the GFP(high) subpopulation may differ from the GFP(low) subpopulations. Consistent with a naïve-like state, data mining of single cell RNAseq datasets²⁴ reveals that the expression level of HERVH in hESCs is correlated with several pluripotency-associated genes, including naïve-associated TFs (Fig. E1e).

To collect uniform GFP(high) and GFP(low) hPSCs, we performed two rounds of FACS (Fig. 4a). We first sorted GFP(+) cells that were further divided into GFP(high) and GFP(low) categories. Strikingly, GFP(high) cells are capable of forming tight, uniformly expressing 3D colonies characteristic of naïve mESCs (Fig. 4a; Supplementary Video S1). In contrast, GFP(low) cells form flat colonies, resembling mouse epiblast stem cells (mEpiSCs) (Fig. 4a). We also observed mosaic colonies. Immunostaining of 3D and chimeric colonies reveals that the NANOG and GFP(high) signals copresent (Supplementary Videos S1-2). Thus, the GFP(high) subpopulation in human pluripotent stem cells are enriched for cells resembling the murine naïve/ground state.

To examine this possibility, GFP(high) vs GFP(low) cells were subjected to expression analyses. qRT-PCR revealed significant up-regulation of naïve-associated TFs⁴⁻⁶ and down-regulation of lineage-commitment genes in GFP(high) vs GFP(low) (Fig. 4b). As in naïve mESCs²⁵ and human ICM²⁶ X chromosomes are activated in GFP(high) hESCs_H9, as evidenced by nearly complete loss of condensed H3K27me3 nuclear foci (Fig. 4d) and low level of XIST expression (Fig 4c). However, nearly 60% GFP(low) hESCs transited from GFP(high) hESCs are marked with condensed H3K27me3 foci or higher density of H3K27me3 in the nucleus (Figs. 4d, E8g). These data are consistent with a naïve-like state for GFP(high) cells and a primed state for GFP(low) cells (one X chromosome inactivated or in process of being inactivated).

GFP(high) cells can be maintained in the modified 2i/LIF medium for a long time, with higher single-cell clonality as well as full pluripotency (Fig. E8a-d). However, GFP(high) and GFP(low) cells have slightly different differentiation potential. When differentiation triggered, certain naïve-associated TFs are maintained at higher levels in GFP(high) naïve-like cells compared with GFP(low), and start their differentiation program with a delay (Figs. E8e-f). Early passage hPSC cultures behave somewhat similarly to GFP(high) cells (Figs. E9a-c).

Transcriptomes of GFP-sorted cell populations and previously characterized naïve-like and primed hPSCs⁴ and mouse counterparts as well as human ICM, support a naïve-like status of GFP(high) cells. Unbiased hierarchical clustering of the expression profiles revealed that GFP(high) and GFP(+) cells have a similar,

but non-identical, expression pattern, one that sharply contrasts with GFP(low) (Fig. E8h). Strikingly, GFP(high) and GFP(+) samples clustered with human ICM and the published naïve-like hPSCs, respectively (Fig. 4e). Importantly, GFP(high) cells cluster closest to human ICM (Fig. 4e).

Cross-species comparison of expression of 9,583 mouse-human orthologs revealed that GFP(high) and GFP(+) correlated to published naïve hPSCs, while GFP(low) clustered with primed cells (Figs. 4f-g), supporting the significance of HERVH-driven transcription defining a naïve-like state.

To address how gene expression changes up to the ICM stage, we analysed 114 RNAseq samples harvested in early developmental stages of embryogenesis²⁴ and 3 RNAseq samples of naïve-like hESCs (3iL_hESC³). HERVH expression appears already in the zygote, but the pattern of activated loci changes during early development (Figs. E9d-e). Importantly, the pattern of active loci characteristic of ICM is the closest to naïve-like hESCs, including GFP(high) (Fig. E9d). Notably, the number of activated HERVH loci is particularly high in hESCs, especially in naïve-like cells and marked with H3K4me3 (Figs. E9d-f), indicating that HERVH may play some roles in the derivation and/or maintenance of naïve-like hPSCs.

To address how HERVH-driven gene expression modulates pluripotency, we surveyed differentially regulated genes in GFP(high) vs GFP(low), intersected by HERVH cis-regulation. The differentially regulated genes located in the neighbourhood (\pm 50 kb) of HERVH display a similar expression pattern to

those differentially expressed in GFP(high) vs GFP(low) and in human naïve-like vs primed stages, derived under specific culture conditions⁴ (Fig. E9h). In contrast, a distinct pattern is observed when comparing mESCs vs mEpiSCs (Fig. E9g). Strikingly, there is an inverse pattern of expression between genes defining naïve-like stage [up in GFP(high) vs GFP(low)] and those that are down-regulated in HERVH knockdowns ($\rho=-0.6$, $P<0.0001$; Fig. E9i), underlying the significance of HERVH in regulating the naïve-like state in humans. Differentially expressed genes between GFP(high) vs GFP(low) populations were enriched for Gene Ontology (GO) terms of developmental processes, morphogenesis and organismal processes (Fig. E9j). Transition of naïve-like cells into primed state following depletion of HERVH supports the above conclusion (Fig E9k).

While GFP(high) cells have many properties resembling naïve mESCs, they are better regarded as being naïve-like, not least because it is unclear that human and naïve mESCs need be identical. Indeed, while LBP9 is associated with pluripotency^{6,20} in mammals, HERVH was recruited to the pluripotency network exclusively in primates. How then to define naïve human pluripotency if we do not necessarily expect them to be identical to mouse ones? We suggest that, rather than hard to replicate inter-species chimaera experiments²⁷, the optimal approach is to define cells by similarity of expression to the ICM (see Supplementary Discussion). In this regard GFP(high) cells are one of the best current models of naïve-like status.

That LBP9 forms heteromer complexes functioning either as a transcriptional activator or a repressor, depending upon the partner²⁸ is consistent with HERVH

being recruited to the pluripotency network by serendipitous modification of a pluripotency factor detailed to defend the cell against it (Fig. E10). Whatever the origin, LTR7/HERVH is an efficient reporter for the naïve-like state most probably because it acts as a platform for multiple key pluripotent transcription factors²⁹. Similarly the LTR7-GFP reporter should enable optimization of naïve-like hPSC culture conditions.

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Author contributions

This project was inspired by MCL. Zlz, LDH and JW conceived ideas for the project, and wrote the manuscript with contributions from other authors. The project was supervised by Zlz and LDH. Zlv provided critical advice. JW designed and performed experiments, analyzed and interpreted data, and participated in bioinformatic analyses. TR contributed by EMSA and assisted in immunostaining experiments. AS assisted in the reporter assays. HC assisted in shRNA cloning. WC and JW performed RNAseq experiments. AP provided materials, and performed karyotype analysis. DB, NVF and GGS provided materials. GX performed RNAseq, bisulfite-seq and ChIP-seq analyses. MS analyzed microarray data and performed cross-species correlation studies. LDH and ATG performed all the other bioinformatic analyses.

RNASeq and microarray data were submitted to NCBI's GEO database at accession GSE54726.

Ethics approval

For work on human ES cells we obtained No.6 allowance from the Robert Koch Institute, Germany (08.10.2004). The human embryonic stem cell lines (H1, H9, BGN1, and BGN2) are permitted to be used in the study "Mechanisms of single transduction in the maintenance of undifferentiated state in human embryonic stem cells."

The author's declare no competing financial interests.

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For online methods and Supplementary Data

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Figure 1. HERVH is a specific marker of human pluripotent stem cells (hPSCs)

a, Expression of various Transposable Elements (TEs) in human induced pluripotent stem cells (hiPSC), hESC (H1), and human fibroblast HFF-1. Colours indicate different classes of TEs (red, long terminal repeat elements (LTR); green, long interspersed nuclear elements (LINE); blue, short interspersed nuclear elements (SINE); grey, other repeat elements). **b**, The proportion of active loci in each HERV family. **c**, Relative mRNA levels of HERV(H/K/W) in hESC (HES-3), various hiPSCs lines and their parental somatic cells. **d**, Effect of long-term culturing on HERVH transcription levels in hiPSCs generated from HFF-1. P, passage number. **c**, **d**, mRNA levels are normalized to GAPDH, and relative to HES-3. Error bars, s.d. (n=3 independent cell cultures), t-test, *P<0.05.

Figure 2. HERVH is recruited into the circuitry of human pluripotency

a, The distribution of H3K4me3 and H3K9m3 in active vs inactive HERVH regions in hiPSCs, hESCs and HFF-1. **b**, Conserved binding sites of OCT4, NANOG, LBP9 and KLF4 are shown in active LTR7s vs moderately active versions of LTR7Y/C. The Jaspar consensus sequence of the LBP9 is shown. **c**, Confirmation of LBP9 binding to LTR7 by ChIP-qPCR with two different primers (LTR7#1, #2) targeting LTR7 regions. HERVH-gag, HERVH-pol and LTR5_Hs (LTR of HERVK) served as negative controls, while an upstream region of NANOG (7.5 kb from TSS) was a positive control. Data are collected from two independent experiments with biological replicates per experiment (LBP9: n=3; IgG: n=2), error bars, s.d.; t-test *P<0.05, **P<0.01. **d**, Upregulation of HERVH transcription in HFF-1 regulated by exogenous pluripotency-associated transcription factors. Data are collected from three independent experiments with biological triplicates per experiment. **e-f**, Effects of shRNA knockdowns of various TFs on HERVH and HERVK transcription in hESC_H9. Data shown are representative of three independent experiments with biological triplicates per experiment. **d-f**, error bars, s.d.; t-test *P<0.05, **P<0.01, P***<0.001.

Figure 3. HERVH triggers pluripotency-regulating hPSC-specific chimeric transcripts and lncRNAs

a, Expression of HERVH forces diversification of transcripts in hPSCs. Left: schematic representation of the HERVH-derived alternative and chimeric transcripts. Right: RT-PCR detects HERVH-specific transcripts (marked by triangles) in hPSCs and NCR1 in embryoid body (EB), but not in HFF-1 or K562. Yellow arrows indicate primer binding sites. **b**, The effects of LBP9 and HERVH-derived transcripts on reprogramming of HFF-1 to hiPSCs. Upper panel: Representative TRA-1-60 stained wells are shown. Lower panel: The number of TRA-1-60⁺ hiPS colonies reprogrammed from HFF-1 by LBP9, ESRRG or LTR7-CD in conjunction with OCT4, SOX2, KLF4 and c-MYC (OSKM). Error bars, s.d., t-test *P<0.05, **P<0.01 from three independent experiments. **c-d**, qRT-PCR analyses to determine the relative expression level of pluripotency and differentiation markers after knockdown of LBP9 (**c**) or HERVH (**d**) in hESC_H9. Data shown are representative of three independent experiments with biological triplicates per experiment. Error bars, s.d., t-test *P<0.05, **P<0.01, and ***P<0.001. ND, not detected. Representative immunostainings show the expression of PAX6 and CDX2 in LBP9 and HERVH knockdowns (scale bar, 100 μ m). **e**, Heat map showing genome-wide gene expression in hESC_H9 following knockdown of GFP (shGFP), LBP9 (shLBP9) and HERVH (shHERVH). The knockdown effect of LBP9 and HERVH are highly similar (rho from Spearman's correlation). For list of affected genes, including direct targets of shHERVH see Tables S13 and S14. **f**, Venn diagram shows that 1094/2627 genes are similarly affected by KD-HERVH and KD-LBP9 (Table S12).

Figure 4. HERVH genetically marks naïve-like hESCs

a, Experimental scheme for isolating naïve-like hPSCs. pT2-LTR7-GFP#2-marked hESC_H9 were enriched by FACS-sorting in multiple rounds and cultured in *conventional hESC medium* and in 2i/LIF medium, respectively. Scale bar, 200 μ m. See also Supplementary Videos S1 and S2. **b**, qRT-PCR analyses of multiple transcription factors and markers for naïve and primed state in GFP(high) and GFP(low) cells, respectively. **c**, qRT-PCR analysis of XIST in GFP(high), GFP(low) hESC_H9 and human female fibroblasts (HLF). **b, c**, Error bars, s.d.; t-test *P<0.05, **P<0.01, and ***P<0.001 (n=3 independent cell cultures). **d**, Representative confocal images obtained after immunostaining for H3K27me3 on GFP(high),

GFP(low), hESC_H9s and HLF. Scale bar, 20 μ m. The proportions of H3K27me3 foci(+) (triangles) and (-) cells in each sample are shown in the histogram. Error bar, s.d.. Data were obtained from 100-450 cells counted from five images per sample. **e**, Global expression cluster dendrogram between GFP(high), GFP(+), GFP(low) hESCs_H9, human inner cell mass (ICM) and previously established human naïve and primed cell lines⁴. Approximately Unbiased (AU) probability, Bootstrap Probability (BP) values and edge numbers at P-value less than 0.01 are shown. ICM clusters closest with GFP(high) – nodes 7,9. **f**, Correlation matrix displaying the unbiased and pairwise comparison of mouse–human orthologous gene expression between GFP-marked hESC_H9 (this study, green) and mouse and human⁴ naïve as well as primed PSCs. Color bar indicates Spearman correlation strength. **g**, Cluster analysis using the average distance method on the same dataset as in **f**. GFP(high), GFP(+) and GFP(low) cells in **e-g** were collected from hESC_H9 cells cultured in *conventional human ESC medium* by FACS-sorting.







